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13. ABSTRACT *(Maximum 200 Words)*

The purpose of our research is to develop better chemotherapeutic drugs for the treatment of prostate cancers. We have used chemical synthetic methods to create bifunctional compounds consisting of a ligand for the androgen receptor linked to a reactive alkylating group that can produce covalent damage in cellular DNA. It is proposed that such damage would persist in tumor cells that express the androgen receptor (AR) because the DNA lesions would be masked by their association with the AR. Initial work prepared chemically modified non-steroid and steroid derivatives that were tested for their affinity for the AR. This work led to the identification of structures that when attached to a linker molecule still retained good affinity for the AR. Subsequently, a number of bifunctional compounds were constructed and tested in biochemical assays and in prostate cancer cells in culture. We have identified a lead compound containing an 11β-substituted steroid linked to an aniline mustard. This compound damaged DNA and retained good affinity for the AR. We discovered, however, that when added to prostate cancer cells in culture its AR binding activity is lost. We are conducting experiments of understand these results and guide us in the preparation of additional compounds.

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(4) TABLE OF CONTENTS

(5) INTRODUCTION:

The objective of our research project is the development of selective genotoxins that can provide more effective treatments for prostate cancers. Our proposal described the chemical synthesis and biological evaluation of bifunctional molecules that produced DNA damage that persists in tumor cells leading to greater toxicity and potentially a higher therapeutic index. To accomplish this goal we have employed chemical synthetic methods to prepare molecules in which a group capable of alkylating DNA is tethered to a ligand for the androgen receptor (AR). In principle the alkylating group of such bifunctional molecules will form covalent DNA adducts that have high affinity for the AR. Adducts engaged in tight complexes with the AR protein will be concealed from proteins that remove and repair such damage to cellular DNA, resulting in greater cytotoxic and therapeutic response in prostate tumors that express high levels of the AR. To accomplish these goals the initial objective of our research is to identify small organic molecules that can be linked to an alkyl chain and function as ligands for the AR. Once such structures have been identified we proposed to combine them with a nitrogen mustard creating bifunctional molecules capable of forming DNA adducts with high affinity for the AR. The toxic effects and therapeutic potential of these new compounds would then be evaluated in cell culture models of prostate cancers.

(6) BODY:

Task 1: *Identify small organic compounds that have high affinity for the AR and discover means by which they can be linked to reactive "warheads," while maintaining good affinity for the AR.*

Synthetic work has been completed on several arylthiohydantion compounds. Our attempts to link an aromatic nitrogen mustard to arylthiohydantoins that reportedly have high affinity for the AR have not met with success. Several phenylthiohydantoins that differed in the electron withdrawing groups attached to the phenyl moiety were linked via an alkylcarbamate to an aniline mustard. The structures of these bifunctional compounds are shown in Figure 1. The affinity for the AR of compounds shown in Figure 1 was assessed using a whole-cell assay in which LNCaP cells in culture were exposed simultaneously to [^{3}H] R1881 and the test compound. Following incubation at 37°C for several hours the amount of [^{3}H] associated with the cells was determined. Neither of these compounds was found to have a good affinity for the AR. Our results indicate that phenylthiohydantoins that bind to the AR are quite sensitive to structural alterations. It is therefore unlikely that they will be useful for our purposes.

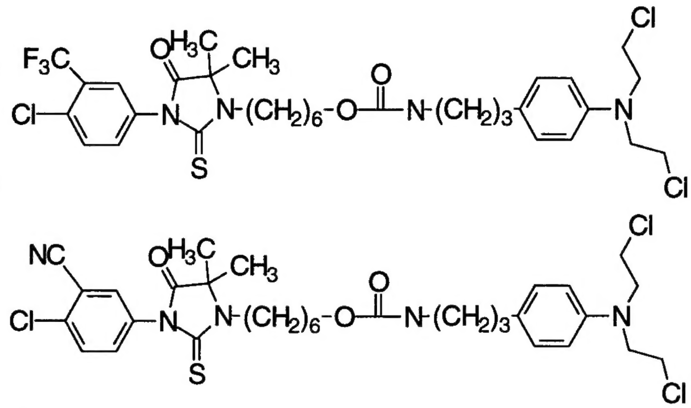

Figure 1. Phenylthiohydantoin linked aniline mustard compounds.

We have also completed synthetic work on a number of phthalimide and benzamide compounds and evaluated them as potential ligands for the AR.

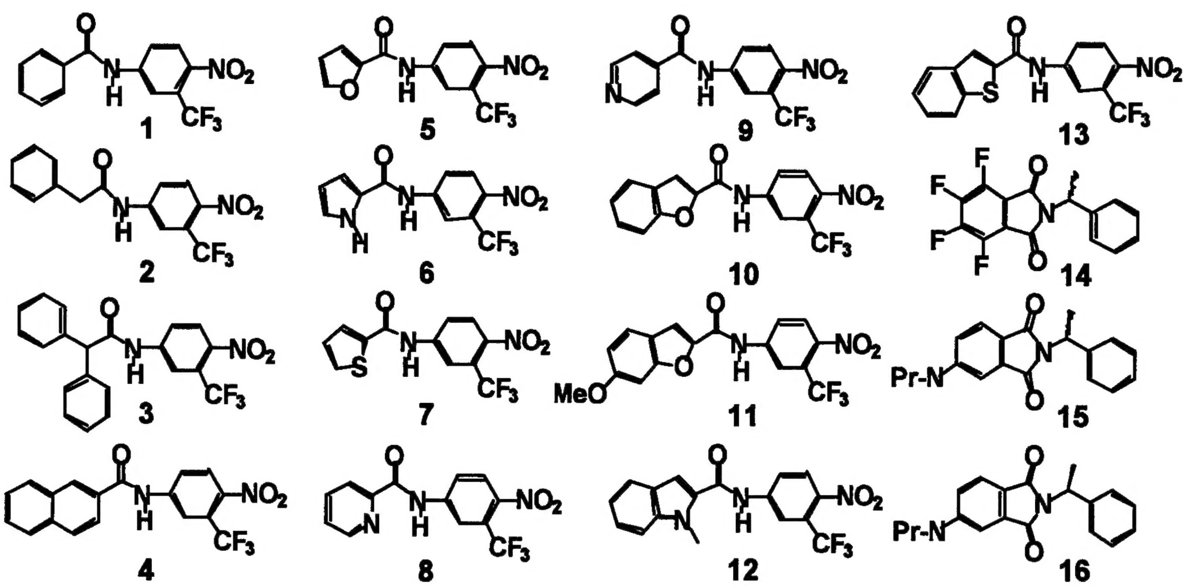

Figure 2. Structures of benzamide and phthalimide compounds that have been synthesized and tested for affinity for the AR.

The EC50s[1] for the benzamide and phthalimide compounds shown in Figure 2 ranged from approximately 8 μM for compounds **5** and **7** to greater than 50 μM for compounds **11**, **15**, and **16**. Because of their relativity low affinity for the AR, we have not pursued incorporation of either the phthalimide or benzamide structures into bifunctional molecules.

Synthetic work in the area of steroid ligands for the AR has produced more promising results. As described in our proposal, in preliminary studies we discovered that dihydrotestosterone (DHT) compounds modified by 6-carbon alkyl groups at the 7α position retained high affinity for the AR. Based on this finding we constructed the bifunctional 7α DHT compound shown in Figure 3. To our surprise competitive binding studies with [3H] R1881 indicated that this compound was inactive as a ligand for the AR – at concentrations up to 2 μM. Negative results in the competition binding assay were also obtained for related compounds in which the length of the alkyl chain between the DTH and secondary amino group was increased to 8 or 10 methylene groups.

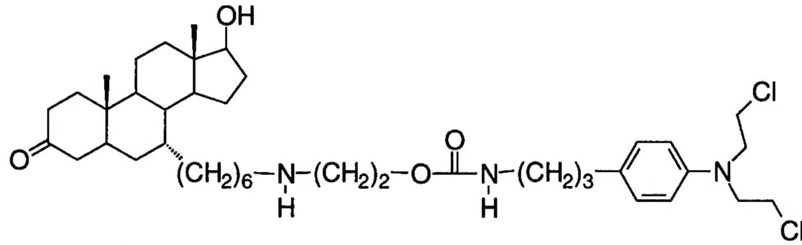

Figure 3. Structure of the 7α-DHT-linked aniline mustard.

We investigated the reason(s) that the bifunctional 7α-DHT compounds were unable to function as ligands for the AR. Since the 6-OH-hexyl-7α-DHT compound did bind well to the AR we speculated that the amino group was somehow interfering with the binding of the bifunctional compounds with the AR. Synthesis of 7α-hexyl-DHT compounds with N,N-dimethylamino-, and pyrrolidino- groups provided evidence that this was indeed the case since unlike the alcohol, neither compound with the substituted amine bound to the receptor. We are now investigating other linker groups that can be incorporated at the 7α position of the DHT molecule while retaining its ability to bind to the AR.

Another group of molecules that have shown even greater promise are those in which have aminoalkyl substituents attached at the 11β position of the steroid skeleton. We began these investigations because of published reports that the antiprogestin RU486 – an 11β-substituted steroid – bound well to the AR. Following published synthetic procedures we prepared 17β-hydroxy-11β-(N,N-dimethylamino-6-hexyl)-4,9-estradien-3-one. Competitive binding studies conducted *in vitro* using extracts obtained from LNCaP cells found that this 11β compound bound well to the AR. These results led us to prepare the 11β-substituted

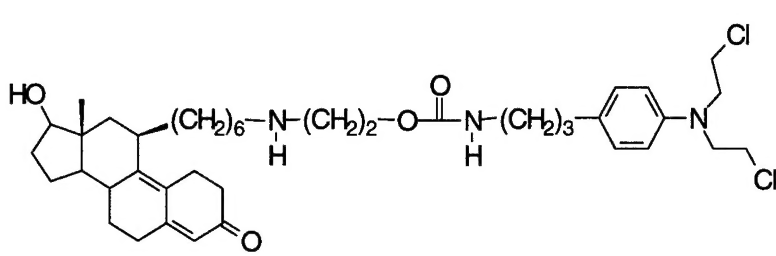

Figure 4. Bifunctional, 11β-4,9-estradien-3-one C6NC2-mustard compound that binds to the AR.

[1] The EC50 is the concentration of the test compound that is required to decrease to 50% the amount of [3H] R1881 bound to the LNCaP cells.

bifunctional compound shown in Figure 4. As shown in Figure 5, the bifunctional mustard also has significant affinity for the AR with an RBA of 5% when compared to R1881. This RBA is comparable to that of a related compound that bound to the estrogen receptor and showed enhanced toxicity toward breast cancer cells that express the estrogen receptor. We were, therefore, optimistic that the 11β compounds would have a modest degree of selective toxicity toward cells lines such as LNCaP and LAPC4 that express the AR.

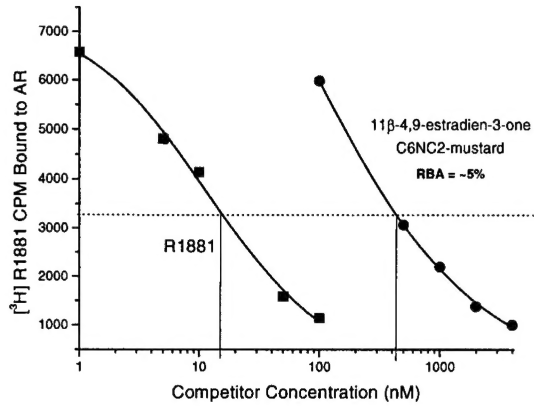

Figure 5. Binding of [3H] R1881 and unlabeled competitors for the AR in LNCaP cell extract.

Task 2: *Construct libraries of bifunctional molecules containing both the AR ligand and the warhead and identify those compounds that can form DNA adducts with high affinity for the AR.*

Progress on this task has been incorporated into the reports on that of Task1 and Task 4. We anticipate that once a lead molecule has been identified that a combinatorial chemical approach to the synthesis of optimized compounds will begin.

Task 3: *Determine whether compounds identified in Aim 2 show enhanced toxicity in prostate cancer cells that express the AR.*

Using a growth inhibition assay as a measure of toxicity we have determined the effects of the compounds described above on various prostate cancer cells in culture. The cell lines used to test the cytotoxic effects include two that express the AR (i.e., LNCaP and LAPC-4) and two that do not express the AR (i.e., DU145 and PC3).

We decided to test whether any of the benzamide or phthalimide compounds shown in Figure 2 had potential therapeutic effects on prostate cancer cell lines resulting from their ability to inhibit growth. Each of the 4 cell lines described above was exposed to concentrations of 2 μM, 10 μM and 50 μM of the compounds shown in Figure 2. After 48 hr the numbers of cells in treated cultures were compared with those in untreated controls. The concentrations of benzamide or phthalimide compounds that inhibited growth by 50% (IC50) are reported in Table 1.

Table 1. IC50 values (μM) for growth inhibition of prostate cancer cell lines by compounds shown in Figure 2.

Compound	PC3	DU145	LNCaP	LAPC4
1	ND†	41	11	50
2	24	ND	ND	44
3	ND	ND	ND	ND
4	8	33	8	26
5	16	50	10	ND
6	13	23	3	12
7	34	33	6	6
8	42	46	11	18
9	26	>50	ND	23
10	24	27	>50	ND
12	19	7	12	66
13	3	7	8	2
15	45	14	10	39
16	50	ND	10	24

† ND = no data

The results in Table 1 indicate that at least one compound selectively inhibits the growth of the two cell lines that express the AR (i.e., LNCaP and LAPC4). Compound 7 was found to have an IC50 of 6 μM for these two cell lines, which is 5-fold lower than that for PC3 (IC50=34 μM) or DU145 (IC50=33μM) cell lines that do not express the AR and are not androgen dependent for growth. Most compounds, however, did not show significant selectivity in their growth inhibitory effects. These results were expected since as reported above (see Task 1) these compounds are – at best – weak ligands for the AR and therefore unlikely to function as receptor antagonists. Thus, compounds that were found to be fairly potent growth inhibitors are likely to act via a different biochemical mechanism. Compound 13, for example, is a benzamide derivative containing the heteroaromatic benzothiophene moiety that exhibited IC50s of <10 μM for all four cell lines. Further investigation of the therapeutic potential of this compound in animal models could be warranted since gram quantities of it can be easily prepared from commercially available starting materials.

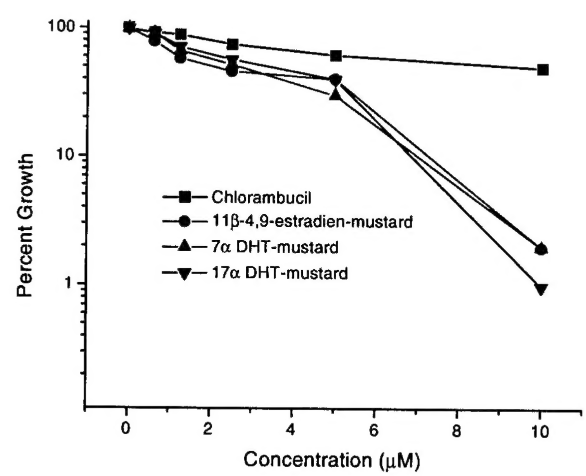

Figure 6. Inhibition of growth of LNCaP cells by bifunctional mustard compounds.

We performed similar growth inhibition experiments to evaluate the cytotoxic effects of the bifunctional compounds that incorporated steroids as potential ligands for the AR. The cytotoxic effects of the 11β-4,9-estradien-mustard compound – that has good affinity for the AR – along with DHT compounds substituted at the 7α-, and 17α-

positions – that are not good ligands for the AR – were tested in the AR+ and AR- prostate cancer cell lines described above. Figure 6 shows the ability of these compounds to inhibit the growth of LNCaP cells. The response of LNCaP cells to chlorambucil – a nitrogen mustard in clinical use – is shown for comparison. The results indicate that all of the bifunctional compounds are more effective than chlorambucil in inhibiting the growth of these cells. Results similar to those depicted in Figure 6 were obtained for identical growth inhibition experiments with LAPC-4, DU145, and PC-3 cell lines. Thus, in these experiments we did not find the selectivity toxicity of the 11β-4,9-estradien-mustard compound in AR+ cell lines (i.e. LAPC-4 and LNCaP) that we had hoped to observe. Nonetheless, all of the new compounds tested are very effective in preventing the growth of the 4 prostate cancer cell lines tested at therapeutically relevant concentrations.

The absence of selective toxicity of the compound incorporating the 11β-substituted 17β-hydroxy-4,9-estradien-3-one could indicate that the 17β-hydroxy-4,9-estradien-3-one ligand was not present or unavailable for binding to the AR at sites of damage in cellular DNA. We are investigating the biochemical basis for these results.

Task 4: *Investigate the biochemical mechanism(s) responsible for selective toxicity.*

Our investigations of the biological effects of the 11β-4,9-estradien-mustard compound were disappointing because of the encouraging results that we had obtained indicating that it had good affinity for the AR. The following experiments were performed to explain these contradictory findings.

The competitive binding assays performed with cell extracts *in vitro* were repeated using intact cells in culture. Unlike the results shown in Figure 5 (above) there was no evidence that under these conditions the 11β-4,9-estradien-mustard compound was able to compete with [^{3}H] R1881. This result could explain the absence of selective toxicity of this compound in AR+ cells. We speculate that metabolism of the compound to an inactive form could be responsible for the loss of its affinity for the AR in cell culture. The oxidation of the 17β hydroxy group is a logical possibility that we are currently investigating. If this is the case it can be prevented by additional modification of the 17 position.

We have also begun to modify DNA in vitro with 11β-4,9-estradien-mustard compound to identify the covalent adducts formed and assess their affinity for the AR.

(7) KEY RESEARCH ACCOMPLISHMENTS

- Chemical synthesis of 16 benzamide and phthalimide compounds. Assessment of their affinity for the androgen receptor and growth inhibitory effects on prostate cancer cell lines.

- Chemical synthesis of bifunctional genotoxins that incorporate 11β-, 7α-, and 17α-substituted steroids as potential ligands for the androgen receptor.

- Identification of 11β-4,9-estradien-mustard as a lead compound. This bifunctional molecule has good affinity for the androgen receptor when assayed in cell extracts *in vitro* while steroids with similar substitutions at the 7α and 17α positions do not.

(8) REPORTABLE OUTCOMES

A manuscript entitled "Novel flutamide and phthalimide derivatives evaluated as androgen receptor ligands", Shimizu, L.S., Croy, R.G., Marquis, J., Mulumba, D. and Essigmann, J.M. is in preparation.

"Programmable therapeutics for prostate malignancies." Essigmann, J.M. and Croy, R.G., Presented at the Fifth Annual CaP-Cure Conference. September, 1998.

(9) CONCLUSIONS

We have made substantial progress in the synthesis and identification of novel molecules – steroidal and non-steroidal – that can function as ligands for the androgen receptor. This work has led to the synthesis of a bifunctional molecule that has good affinity for the AR in cell extracts *in vitro*, but loses this essential property when assayed cells in culture. This is likely the reason for the absence of selective cytotoxicity of this compound in prostate cancer cells that express the AR. We are now engaged in experiments that we anticipate will explain this result. It will then be possible to chemically modify our lead compound to prevent loss of its ability to bind to the AR. With the structures of active pharmacophores of our molecules established we will then proceed to further structure-activity studies to optimize their AR affinity, ability to alkylate DNA, and selective toxicity toward prostate cancer cells that express the AR.

(10) REFERENCES

None cited.

(11) APPENDICES

No appended material included.